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(54) Title: PHOSPHOETHANOLAMINE FOR TREATMENT OF ALZHEIMER'S DISEASE

(57) Abstract

The present invention is based on the discovery that phosphoethanolamine, a natural product isolated from the brains of animals, and related compounds are cholinergic factors in that treatment of explant cultures of medial septal nuclei with phosphoethanolamine or related compounds results in an increased capacity of these cultures to synthesize the neurotransmitter acetylcholine. The invention provides pharmaceutical compositions comprising ethanolamine or related compounds and derivatives thereof for use in the treatment of Alzheimer's disease.

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Phosphoethanolamine for treatment of Alzheimer's disease

Field of the Invention

The field of the invention is providing pharmaceutical compositions for the treatment of neurological disorders associated with dementia, such as Alzheimer's disease.

Background of the Invention

The causes of some of the most common and most devastating diseases of the nervous system remain unknown. Prominent on this list are amyotrophic lateral sclerosis (ALS), parkinsonism, and Alzheimer's disease. Each of these conditions is presently considered to be a degenerative disorder of unknown origin. In each, viral or immunological causes have been suggested, but no convincing reproducible data support the presence of an infectious agent or a cell-mediated or humoral immune factor. All three diseases reflect pathological change in a relatively limited network within the peripheral or central nervous system, or both.

Alzheimer's Disease

Alzheimer's disease is a disorder of the later decades of life characterized by dementia. In clinical terms, it consists of a diffuse deterioration of mental function, primarily in thought and memory, and secondarily in feeling and conduct. Alzheimer's disease has been used

to designate dementia appearing before the age of 65 years. When the syndrome presents after that age, the term senile dementia of the Alzheimer's type is used. In fact, it appears reasonable to consider both types as representing a single syndrome. The true incidence of the disorder is unknown, although recent data suggest that the incidence of all dementia in the U.S. population may be over 100 cases per 100,000, with its prevalence being over 550 per 100,000 [1]. Alzheimer's disease probably affects at least 30 to 50% of patients with dementia, and in the United States there may be over one million individuals with severe dementia and several million more with mild to moderate dementia. It has been estimated that 1 out of every 6 persons over the age of 65 in the United States suffers from moderate dementia, and a majority of patients in the nursing home populations are affected with the disorder. The average age of onset is between 70 and 79 years, but without better information on the population at risk, a more accurate statement is not presently possible [1]. As in ALS and parkinsonism, the incidence of the syndrome clearly increases with advancing age. A family history of Alzheimer's disease is present in 5 to 10% of the patients.

At the present time, the clinical diagnosis of Alzheimer's disease is one of exclusion. Secondary causes of loss of memory and impaired cognitive function may result from multiple infarcts, leading to so-called multinfarct dementia, or from intracranial mass lesions such as subdural hematomas, brain tumors, or granulomas. Central nervous system infections of viral and bacterial origin, or even slow viral disorders such as Jakob-Creutzfeldt disease, are part of the differential diagnosis. Furthermore, metabolic disorders involving vitamin B_{12} metabolism, thiamine or folate deficiency, thyroid dysfunction, hepatic and renal failure, as well as

drug toxicity, may present as dementia. Nevertheless, when all these secondary causes, many of which are reversible, are eliminated, cerebral atrophy of unknown cause or Alzheimer's disease still covers the largest number of patients. Elevations of aluminum content in the brain have been implicated in the pathogenesis of the disorder but appear to be secondary rather than primary [2, 3].

The pathological picture of Alzheimer's disease has been well characterized over the years. It consists of senile plaques, which result from degeneration of nerve endings, and neurofibrillary tangles, which represent an alteration in the cytoskeletal apparatus [4]. In addition, intracellular cytoplasmic eosinophilic inclusions, termed Hirano bodies, are present, primarily in the hippocampus. Granulovacuolar degeneration is also noted. Senile plaques and neurofibrillary tangles in the brain are part of the "normal" aging process. However, at any age, patients with clinical Alzheimer's disease appear to have much higher concentration of these abnormalities than do normal individuals [5].

A prominent finding in Alzheimer's disease is a deficiency of the enzyme that synthesizes the neurotransmitter acetylcholine, namely, choline acetyltransferase (CAT) [6]. This deficiency is most marked in the cortex and hippocampus. Of note is the fact that acetylcholine receptors in the brain are either unaffected or relatively less affected. Thus, the defect in CAT reflects an alteration in the presynaptic cholinergic neuron. The diminution in CAT correlates with the presence of senile plaques: the greater the number of plaques, the lower the activity of CAT. Enzymes synthesizing several other neurotransmitters, including dopamine, norepinephrine, serotonin, and gamma-aminobutyric acid, as well as levels of vasoactive intestinal peptide, are all relatively unaffected compared

to the loss of CAT activity. Somatostatin-like activity has recently been reported to be decreased in the cerebral cortex [7].

The CAT activity found in the hippocampus appears to derive largely from nerve terminals for which the cell of origin is in the septal nucleus. In addition, almost 70% of CAT activity in the cortex appears to reside in terminals with cell bodies located in the nucleus basalis of Meynert [8]. In rats, these cholinergic neurons lie intermingled with and beneath the medial globus pallidus, whereas in primates comparable cells are found exclusively outside the pallidum. In humans, the nucleus basalis of Meynert is situated in the fibrous zone beneath the globus pallidus and is a major component of the substantial innominata [9]. Thus, the cholinergic input to hippocampus and cortex may derive from a group of cells extending from the septal nuclei to constituents of the substantia innominata and may well be impaired in Alzheimer's disease [9].

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U.S. Patent 4,294,818 discloses a diagnostic method for multiple sclerosis comprised of antibody preparations reactive with antigenic substances associated with lymphocytes.

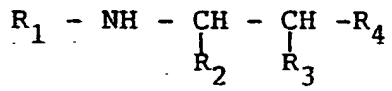
U.S. Patent 3,864,481 discloses a synthetic amino acid for suppression and diagnosis of multiple sclerosis.

U.S. Patents 3,961,894; 4,046,870; and 4,225,576 disclose assay techniques for detecting hormones in the body.

Summary of the Invention

The present invention is based upon the discovery that phosphoethanolamine, a natural product isolated from the brains of animals, and related compounds are cholinergic factors in that treatment of explant cultures of medial septal nuclei with phosphoethanolamine or related compounds results in an increased capacity of these cultures to synthesize the neurotransmitter acetylcholine. Treatment of Alzheimer's disease with cholinergic factors is based upon the ability of these compounds to improve the function of acetylcholine producing neurons which are impaired in Alzheimer's disease.

The present invention discloses a method of treating a patient having a neurological disorder associated with dementia of the Alzheimer's type comprising administering to said patient an amount of a composition effective in amelioration of the dementia comprising a pharmaceutically acceptable excipient and an active sterioisomeric form of a compound having the structure



or the pharmaceutically acceptable salt thereof,

wherein R_1 is hydrogen or a lower alkyl;

the R_2 and R_3 groups may each be hydrogen, or different groups selected from the group consisting of hydrogen, the lower alkyls, and -COOM groups wherein M is hydrogen or a pharmaceutically acceptable cation; and

R_4 is selected from the group consisting of -OH, $-PO_3H_2$, $-OPO_3H_2$, cytidine 5'-diphosphate, and their pharmaceutically acceptable salts.

Accordingly, it is an object of the present invention to provide an effective treatment for patients suffering from central nervous system disorders, especially those connected with cerebral senescence, such as Alzheimer's disease.

Patients are treated by administering an effective amount of an active sterioisomeric form of a compound of the present invention which is in admixture with a pharmaceutically acceptable excipient.

A further object of the present invention is to provide numerous compounds which are effective in increasing the synthesis of acetyltransferase, and therefore are available for use in the treatment of neurological disorders.

Other and further objects, features and advantages of the invention are set forth throughout the specification and claims.

Brief Description of the Drawing

Figure 1 depicts a flow chart for the purification of the central cholinergic factor isolated from brain extracts.

Figure 2 is an illustration of a mass spectrometric analysis of the phenylisothiocyanate derivatives of a central cholinergic factor isolated from cortical and hippocampal tissues.

Detailed Description of the Invention

From the foregoing, several neurological diseases can be seen to represent disorders of specific neuronal networks; that is, the motor neuronal system, the nigrostriatal neuronal system and the cholinergic neuronal system. These neurological diseases reflect changes in a presynaptic neuronal input with secondary alterations of the target tissue. ALS represents pathological change in Betz cells, cranial motor neurons, and anterior horn cells; parkinsonism, in substantial nigra neurons; and Alzheimer's disease, in the cholinergic input from nucleus basalis and septal neurons to cortex and hippocampus, respectively.

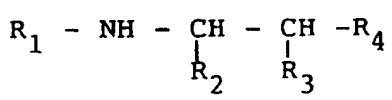
The role of the compositions of the present invention is a modification of the notion of intrinsic aging of

selected neurons; that is, the presence of specific extrinsic factors influence the maintenance and survival of neurons. In many neurological diseases, the system degeneration is due to diminished availability of a specific neurotrophic factor normally released by the post-synaptic cell, taken up by the presynaptic terminal, and exerting its effect by retrograde transport up the presynaptic axon to the soma and nucleus.

For Alzheimer's disease, cholinergic factors can be demonstrated in vitro which increase the activity of the neurotransmitter synthesis enzymes in the innervating cell. The same factors responsible for increasing neurotransmitter synthesis in vitro may also be responsible for increasing neurotransmitter synthesis in vivo. A well-defined cholinergic system in the rat brain and one that is analogous to the neuronal networks affected in Alzheimer's disease is the projection from the medial septal nucleus to the hippocampus. The septo-hippocampal system has been extensively studied with respect to its anatomic relations, developmental neurogenesis, neurotransmitter distribution and capacity for regeneration. Explant cultures of the medial septal nucleus are organotypic such that the associations with glia and other neurons are maintained in the microenvironment of the cholinergic neurons. The in vitro development of cholinergic parameters in such cultures may therefore closely resemble those which normally occur in vivo. Biological compounds added to these cultures which result in the enhancement of these properties would be expected to exist outside the immediate embryonic environment of these neurons. These cultures are therefore suitable for observing the pharmacologic effect of putative cholinergic factors on these cultures. Similar or related factors may also be indirectly responsible for maintenance of neurons throughout the life cycle in vivo, and may decrease as a normal function of aging.

Thus, a primary manifestation of ALS, Parkinson disease, or Alzheimer's disease is failure of the target tissue to supply the necessary neurotrophic factor. Marked pathological change in the tissue need not be present. Impaired synthesis or release (or both) of the relevant hormone would represent the sine qua non of disease. For example, in Alzheimer's disease, the failure would be in hippocampus and cortical cell to supply the relevant cholinergic neurotrophic factor. Thus, in this system, the lack of an appropriate factor released from post-synaptic cells impairs the viability of the presynaptic cells and leads to the gradual deterioration of septal and basal nuclei. With the availability of tissue culture, the presence, deficiency, or absence of specific neurotrophic factors can be assessed in ALS, parkinsonism, and Alzheimer's disease readily and easily and compounds can be identified which are effective to increase the synthesis of such neurotrophic factors for the treatment of Alzheimer's disease.

The present invention discloses a pharmaceutical composition for the treatment of a patient having a neurological disorder asociated with dementia of the Alzheimer's type wherein said pharmaceutical composition comprises a pharmaceutically acceptable excipient and an effective amount of an active sterioisomeric form of a compound having the structure



or the pharmaceutically acceptable salt thereof,

wherein R_1 is hydrogen or a lower alkyl;

the R_2 and R_3 groups may be the same when hydrogen, or different and are selected from the group consisting of hydrogen, the lower alkyls, and -COOM groups wherein M is hydrogen or a pharmaceutically acceptable cation; and

R_4 is selected from the group consisting of -OH, $-PO_3H_2$, $-OPO_3H_2$, cytidine 5'-diphosphate, and their pharmaceutically acceptable salts.

Also disclosed is a method of treating a patient having a neurological disorder associated with dementia which method comprises administering to said patient an effective dementia-treating amount of the pharmaceutical composition of the invention. Reference will be made in this invention to a number of terms which shall be defined to have the following meanings:

"Lower alkyl" means a branched or unbranched saturated hydrocarbon group of one to eight carbon atoms such as, methyl, ethyl, i-propyl and n-butyl and the like.

"Pharmaceutically acceptable cations or anions", or "pharmaceutically acceptable salts" refers to any cation or anion which is pharmaceutically consistent with the mode of administration and does not produce any untoward pharmaceutical effects. Thus, cations may include ions of alkali metals and alkaline earth and transition metals such as calcium, barium, magnesium, sodium, zinc and potassium; and anions may include ions of the mineral acids such as halides, nitrates, sulfates, or phosphates as well as anions of organic acids such as acetate and gluconate.

"Pharmacological precursor" means any biological precursor of the present compounds set forth in the structural formula given in the Summary of the Invention which, upon breakdown of the precursor by normal biological processes, releases serine, phosphoserine, ethanolamine or phosphoethanolamine such that an increase in the levels of these latter compounds in the central nervous system occurs.

"Active sterioisomeric form" of the present invention is intended to include racemic mixtures composed of varying concentrations of both active and inactive racemates but in such ratios that the overall racemic mixture is effec-

tive in stimulating acetylcholine sythesis in cultures of septal neurons, an index of cholinergic maturation.

Many of the compositions of the invention which are set forth in the structural formula given in the Summary of the Invention are well known and are commercially available. For example, phosphoethanolamine, phosphoserine, CDP-ethanolamine and ethanolamine are available from Sigma (St. Louis, MO). Monomethylethanolamine and the active propanol compounds are available from Aldrich Chemical Co., Inc. (Milwaukee, WI).

The R_4 derivatives of ethanolamine and serine of the present invention are commercially available and can be used to construct the R_1 substituted compounds disclosed in this invention by reaction with activated alkyl and activated amino acid derivatives. Thus, R_1 substituted compounds used in this invention can be prepared as follows.

Where R_1 is an alkyl group, the compound is obtained through one of numerous procedures for N-alkylation that are well known in the art. For example, alkyl halides react readily with amines to yield N-alkylated derivatives. Therefore, the reaction of R_1-X wherein X is a halogen such as Br or Cl with the appropriate R_4 substituted ethanolamine or serine results in the R_1-N derivative. Another method is reductive alkylation in which the appropriate activated carboxylic acid derivative of the alkyl reacts with an amino group resulting in the amide condensation product which may then be reduced to give the R_1-N derivative.

In cases where such reactions may modify the R_4 substituent of the compound, the R_1 derivatives may be produced first followed by esterification to yield the appropriate activated R_4 group. Methods for activating phosphate groups in order to obtain phosphate esters by condensation with alcohols are well known in the art. Alternatively, the reactive group on the R_4 substituent may

be protected during the alkylation reaction, for example, by derivatization with a tertbutyloxycarbonyl group.

Surprisingly, phosphoethanolamine has been identified as a brain derived cholinergic factor present in cortical and hippocampal tissues. The discovery that phosphoethanolamine is effective in the treatment of Alzheimer's disease was made through efforts to isolate cholinergic factors from the target regions of cholinergic innervation in the brain, such as cortical and hippocampal brain tissues. Phosphoethanolamine was isolated using the purification scheme outlined in Figure 1 and assaying for stimulation of acetylcholine synthesis in the appropriate assay system described below.

Generally, brain tissue containing the cortex, hippocampus and striatum from young rats and/or calves was homogenized in a buffer solution such as phosphate-buffered saline (PBS) and centrifuged to yield a crude extract. The supernatant was acidified using acetic acid (1 to 2 M) and then recentrifuged.

Since the factor isolated by the process described herein is relatively small (<1,500 daltons), undesired proteins can be removed by ultrafiltration of the extract through an appropriate filter, for example, an Amicon YM-5 filter. Alternatively or conjunctively, the extracted factor may be directly purified by gel filtration chromatography using an appropriate matrix, such as a Biogel P-2 polyacrylamide column, to resolve proteins having molecular weights less than 1,500 daltons.

Several fractionation procedures which can be used singly or in combination to increase purity of a composition are well known in the art. These include size fractionation using molecular sieve (or gel filtration) chromatography, ion exchange chromatography under suitable conditions; affinity chromatography using, for example, antibodies directed to the biologically active form of the neurotrophic factor; absorp-

tion chromatography using nonspecific supports, such as hydroxyapatite, silica, alumia, and so forth; and also gel-supported electrophoresis.

In the present invention, the cholinergic factor, in fractions corresponding to an apparent molecular weigh to the ~1,000 daltons, is eluted from a P-2 gel filtration column. The cholinergic factor is then bound to an anion exchange column (AG-1-X2) at ~pH 8.5 in low salt and then eluted with a low pH (pH 5.5), high ionic strength buffer. Final purification can be achieved by cycling and recycling over different reverse phase matrices which effectively remove most protein since the active factor does not bind to these matrices in 0.1% trifluoroacetic acid (TFA).

The ~1,000 dalton cholinergic factor was assayed on explant cultures of septal neurons obtained from mammalian species. It is preferred to use explants of the medial septal nuclei obtained from the forebrains of E16 rat embryos. The preferred method of assay is to incubate explants of rat septal nuclei with or without the cholinergic compounds for 6 days and then to measure the effect of the compound on the ability of the cells to synthesize acetyl-choline. Assays employing survival, cell growth or the enhancement of other cholinergic properties in this and other types of cultures can also be used to assay for these neurotrophic compounds.

Chromatographic and mass spectroscopic analyses of the central cholinergic factor prepared as outlined above indicate that the main component present is the known compound, phosphoethanolamine. The identity of phosphoethanolamine as the active component of the central cholinergic factor (C-CF) preparation was established by testing pure phosphoethanolamine in the above tissue culture assay for cholinergic factors. Phosphoethanolamine was found to be active at concentrations corresponding to the phosphoethanolamine levels in the C-CF preparation. Several

other related compounds have also been found to be active including certain propanolamines.

Phosphoethanolamine has been purified from the target regions of cholinergic innervation in the rat and has been shown to enhance the ability of medial septal explants to synthesize acetylcholine, an index of cholinergic maturation. Since phosphoethanolamine is effective in enhancing the in vitro cholinergic properties of neurons, the exogenous administration of this and related compounds may be an effective therapy in cases of Alzheimer's disease and general aging of the nervous system if cholinergic function is enhanced in vivo.

The formulations of this invention are useful for parenteral administration, for example, intravenous, subcutaneous, intramuscular, intraorbital, ophthalmic, intraventricular, intracranial, intracapsular, intraspinal, intrasternal, topical, intranasal aerosol, scarification, and also for oral administration. The compositions can be formulated for parenteral administration to humans or other mammals in therapeutically effective amounts (e.g., amounts which eliminate or reduce the patient's pathological condition) to provide therapy for Alzheimer's disease.

The compounds provided herein can be formulated into pharmaceutical compositions by admixture with pharmaceutically acceptable nontoxic excipients and carriers. As mentioned above, such compositions may be prepared for use for parenteral (subcutaneous, intramuscular, intraspinal, or intravenous) administration particularly in the form of liquid solutions or suspensions; for oral administration, particularly in the form of tablets or capsules; or intranasally, particularly in the form of powders, nasal drops or aerosols.

The compositions may conveniently be administered in unit dosage form and may be prepared by any of the methods well known in the pharmaceutical art, for example, as

described in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA, 1970. Formulations for parenteral administration may contain as common excipients sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes and the like. Formulations for inhalation administration contain as excipients, for example, lactose or may be aqueous, such as glycocholate and deoxycholate, or oily solutions for administration in the form of nasal drops.

The materials of this invention can be employed as the sole active agent in a pharmaceutical or can be used in combination with other active ingredients.

The concentration of the compounds described herein in a therapeutic composition will vary depending on a number of factors, including the dosage of the drug to be administered, the chemical characteristics, e.g., hydrophobicity of the compounds employed, and the mode of administration. In general terms, the neurotrophic compounds are provided in an aqueous physiological buffer solution containing about 0.1 to 10% w/v factor for parenteral administration. Typical dose ranges are from about 10 ug/kg to about 1 g/kg of body weight per day; a preferred dose range is from about 1 mg/kg to 100 mg/kg of body weight per day.

The present invention will be further illustrated by the following examples. These examples are not to be construed as limiting the scope of the invention, which is to be determined solely by the appended claims.

EXAMPLE 1

Preparation of Central Cholinergic Factor (C-CF)

Initial Extraction and Purification from Rat Brain

Brain tissue containing the cortex, hippocampus and striatum was dissected from 2-3 week old Sprague-Dawley rat pups, homogenized in 5 volumes of ice-cold phosphate buffered saline (140 mM NaCl/ 2.6 mM KCl/ 1.4 mM KH_2PO_4/ 1.2 mM $NaHPO_4$, pH 7.2) and centrifuged for 90 minutes at 100,000 x g. The resulting supernatant was acidified to 2 M acetic acid and again subjected to ultracentrifugation. The acidified supernatant was filtered through an Amicon YM-5 membrane. The filtrate was lyophilized, redissolved in 0.2 M acetic acid (0.05 times the original volume), filtered and applied to a 2.5 x 100 cm Bio-Rad P-2 column for chromatography in the same buffer. The elution profile was monitored at 0.D.280, and a peak with an apparent molecular weight of 1000 daltons was collected, lyophilized and redissolved in a buffer containing 130 mM N-ethylmorpholine, 250 mM pyridine, and 17 mM acetic acid, pH 8.3 (Buffer A). Approximately 50 mg protein [measured by Lowry's method (Lowry, O.H. et al., J.Biol.Chem. (1951) 193: 265-275)] was applied to a 1.5 x 30 cm Dowex AG 1-X2 (Bio-Rad) anion exchange column (converted to the acetate form) and washed with 250 ml of buffer A. The column was developed with a linear gradient from buffer A to 1.0 M acetic acid (125 ml each buffer), pH 5.5, to form a decreasing pH gradient. The column profile was monitored by reaction with fluorescamine according to the method of Udenfriend (Udenfriend, S., et al., Science (1972) 178: 871-872), after which fractions were pooled, lyophilized and redissolved for assays of bioactivity.

Reverse-Phase HPLC

Active samples were dissolved in 0.05% trifluoroacetic acid (TFA) and injected (150 - 200 ug/injection) onto a Waters C-18 uBondapak (3.9 mm x 30 cm) column equilibrated with the same buffer at a flow rate of 0.5 ml/minute. The chromatogram was developed isocratically and peaks detected at O.D.214 were collected, lyophilized and tested for activity. The active material was redissolved in 0.05% TFA and recycled three times over a Spherisorb O.D.S. II (6.2 mm x 15 cm) column in the same buffer. The purification scheme is outlined in Figure 1 of the accompanying drawings.

The purification results in Table 1 demonstrate that these steps effect a greater than 2500-fold purification from the YM-5 filtrate based on protein content of HPLC fractions measured at an absorbance of 214. This was equivalent to a more than 1 x 10^6-fold purification based on gram wet weight starting material. About 250 rat brains (~70 g cortex/hippocampus) yielded about 100 ug of phosphoethanolamine.

Table 1

Fraction	Specific Activity	Total UG Protein	Total Units	Fold Purified	Units Gm Brain	% Recovery
YM-5 FILTRATE	0.346	43375	15008	1	200.1	100
P-2	3.33	3000	10000	9.6	133.3	66.6
DOWEX	6.72	1120	7526	19.4	100.3	50.1
uBONDAPAK	55.5	80	4440	160.4	59.2	29.6
SPHERISORB	100	20	2000	2890.2	26.7	13.3

Example 2

Preparation of Central Cholinergic Factor (C-CF)

Initial Extraction and Purification from Calf Brain

All operations through the molecular sieving step were carried out in the cold. Calf brains from six month or younger animals were purchased as frozen tissue and stored at -80°C until use. Five brains were allowed to partially thaw at room temperature for one hour, at which time the cerebellum, brain stem and thalamus area were removed. The remaining material was chopped and processed in 300 g amounts by grinding in a blender with an equal volume per weight 1 M acetic acid for three minutes. The homogenate was centrifuged at 28,000 x g for 20 minutes in a Sorvall GSA rotor to yield a crude supernatant. This supernatant was passed through an Amicon YM-5 ultrafilter overnight, and the resulting filtrate was lyophilized to dryness.

The lyophilized extract was resuspended in 50 ml/ 0.2 M acetic acid, and a slight insoluble residue removed by filtration through a 0.45 u filter prior to application on a P-2 polyacrylamide molecular sieving column. Molecular sieving was accomplished on a 5 x 140 cm column (Bio-Rad) eluted with 0.2 M acetic acid at a linear flow rate of 5 cm/hr. Fractions were collected only in the molecular weight range, including the void volume, through 700 mw. The fractions were assayed in the tissue culture system outlined below. The peak of activity migrating near 1000 mw was pooled and lyophilized to dryness.

A 1.2 L Dowex 1 x 2 column was equilibrated in 1.5% N-ethylmorpholine, 2% pyridine titrated to pH 8.5 with acetic acid. The lyophilized active fraction from the molecular sieving step was applied in 100 ml of the starting buffer and the column was eluted with 2 L of the same buffer at a linear flow rate of 30 cm/hr. Finally, the activity was eluted with 2 L 0.1 M acetic acid titrated to pH 5.5 with pyridine and collected in 10 ml fractions. The fractions surrounding the

first fluorescamine reactive peak were assayed as described below for ChAT stimulating activity. The activity fractions were pooled and lyophilized to dryness and further purified by HPLC as described above for the rat brain derived factor.

Example 3

Assay Method for Stimulation of Acetylcholine Synthesis In Explants of the Medial Septal Nucleus

Whole septal nuclei were dissected from the forebrains of 16 day old rat embryos (ED 0 being the day of sperm positivity). The dorsal, caudal and lateral tissues were removed and the remaining medial fragments were sectioned into 0.3 mm pieces by pressing a nickel grid (Ladd Research, #10080) over them. Approximately 20 to 30 explants were plated on poly-lysine coated, 35 mm culture dishes in 1.5 ml of a modified N_2 defined medium consisting of insulin (5 ug/ml), transferrin (100 ug/ml), putrescine (100 uM), progesterone (20 nM), selenium (30 nM), glutamine (4 mM), vitamin B-12 (350 nM) and gentamicin (50 ug/ml) in high glucose (0.6%) DMEM. Cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. Cultures were fed two days after plating by removing 0.5 ml of the plating medium and adding 1.0 ml of fresh medium containing a 2% rabbit serum supplement and the substances being tested. Partially purified fractions were lyophilized and redissolved at concentrations ranging from 10 ng to 1 ug protein per ml. Phosphoethanolamine purified from rat brain and authentic compounds purchased from Sigma Chemical Co. (St. Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI) including those represented in Table 2, as well as, phosphocholine, choline, dipalmitoyl-phosphatidylethanolamine and dipalmitoyl-phosphatidycholine. These compounds were tested at concentrations ranging from 10^{-6} to 10^{-4} molar. Cells were again fed three days later in the same fashion replacing 1.0

ml of old medium with fresh medium containing 1.0% serum and added components.

After three more days in culture acetycholine synthesis was measured by the method of Johnson and Pilar [J. Physiol. (London) 299:605 (1980)]. Cultured explants were washed in 2.0 ml of a Tyrode's type buffer containing 150 mM NaCl, 3 mM KCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, 12.2 mM glucose and 10 mM Hepes, pH 7.4, preincubated in the same buffer for 10 minutes at 37°C, and then incubated in the same buffer containing 0.5 uM choline chloride and 6 uCi [3H] choline chloride for 40 minutes at 37°C. Cultures were then cooled on ice for 10 minutes and washed three times with 2.0 ml ice-cold buffer without $CaCl_2$. Both [3H] choline and newly synthesized [3H] ACh were extracted from the tissue in 0.6 ml of 1 M formic acid/acetone, 15:85 (vol/vol) containing 3.0 nM [14C] ACh and dried under vacuum over phosphorous pentoxide and NaOH. The free [3H] choline was phosphorylated with choline kinase and the [3H] ACh and [14C] ACh were extracted into toluene scintillation fluid with tetraphenylboron by the method of Rand and Johnson [Analytical Biochem 116:361 (1981)]. Total [3H] dpm was calculated relative to the recovery of [14C] dpm and the net [3H] dpm was determined by subtracting the [3H] dpm recovered from culture dishes without explants.

The activity of a compound was measured by calculating the increase in [3H] acetylcholine formed per explant as compared to that of nontreated control cultures. A unit of activity was defined as the amount of material needed to give a stimulation equal to 50% of the maximum response, which was typically a 2-fold increase over controls. Phosphoethanolamine gave a maximal saturating response at 3×10^{-5} molar, thereby exhibiting an ~2.2 fold increase over controls.

Example 4

Structure Determination

The highly purified rC-CF (rat) was analyzed for amino acid content. The results suggested the presence of an unexpectedly large amount of a compound with molecular weight of 141. The only additional component present had a molecular weight of 531 and was a minor component. Amino acid analysis of a lesser amount of material indicated the presence of a very large amount of a primary amine which was not a known amino acid. Based on the absorption of the phenylisothiocyanate (PITC) derivative, there were ~8 micromoles of this amine in the entire preparation from 250 brains.

Mass spectrometric analysis of the isolated PITC derivatives is shown in Figure 2. The combined data are consistent with the compound being phosphoethanolamine (O-phosphoryl ethanolamine), which has a molecular weight of 141. Figure 2A depicts the mass spectrometric profile of the isolated PITC derivatives of acid hydrolyzed rC-CF. Acid hydrolysis of phosphoethanolamine should yield ethanolamine, which would react with PITC to generate a derivative of mass 195 daltons. In fact, a (protonated) molecular ion of 196 is actually observed in field desorption mass spectroscopy. Figure 2B depicts the mass spectrometric profile of the purified PITC derivative of rC-CF without acid hydrolysis. Several mass ions are present which are all consistent with the PITC derivative of phosphoethanolamine; 277 (PITC of phosphethanolamine), 179 (conversion to PITC-ethylenimine), 99 (phosphoric acid), and 197 (diphosphoric acid).

Example 5

Properties of Phosphoethanolamine

In order to confirm the identity of rC-CF as phosphoethanolamine, pure phosphoethanolamine (Sigma Chemical Company) was compared to rC-CF and to cC-CF (calf) obtained in a similar manner as rC-CF. Retention times and profiles obtained by HPLC analysis of the PITC derivatives before and after acid hydrolysis were compared. Phosphoethanolamine, rC-CF and cC-CF had virtually identical profiles by these analyses except that cC-CF appeared to be slightly less pure. Derivatives of phosphoethanolamine and rC-CF coeluted when mixed prior to injection. Even the minor components observed for the C-CFs after hydrolysis were present in the commercial sample after hydrolysis and are therefore intrinsic hydrolysis products.

Example 6

Activities of Compound Analogs

Compounds related to phosphoethanolamine were assayed in substantial accordance with the teaching of Example 3. Cultures of medial septal explants, which had been treated for six days with two additions of the compound of interest, were examined for their ability to convert choline to acetylcholine. A dose-response curved (1 uM to 1 mM) was generated for each compound to determine the effect on acetylcholine synthesis. The results of these experiments are provided in Table 2. As used therein, 1 unit of activity is equal to the molar concentration of the compound required to enhance acetylcholine synthesis 2-fold relative to untreated controls. EC_{50} is the molar concentration at which 50% of the maximum stimulation is induced.

Table 2

Compound	EC_{50}	Molar Concentration Unit Activity
ethanolamine $NH_2CH_2CH_2OH$	5 uM	10 uM
phosphoethanolamine $NH_2CH_2CH_2OPO_3H_2$	.5 uM	10 uM
CDP-ethanolamine $NH_2CH_2CH_2O$-CDP	50 uM	30 uM
dimethylethanolamine $(CH_3)_2NCH_2CH_2OH$	-	-
2-(methylamino)ethanol $CH_3NHCH_2CH_2OH$	1 uM[a]	3 uM
taurine $NH_2CH_2CH_2SO_3$	-	-
phosphonoethanolamine $NH_2CH_2CH_2PO_3H_2$	300 uM	500 uM
2-aminoethyl hydrogen sulfate $NH_2CH_2CH_2OSO_3H_2$	-	-
choline $(CH_3)_3NCH_2CH_2OH$	-	-
O-phosphoryl choline $(CH_3)_3NCH_2CH_2OPO_3H_2$	-	-
CDP-choline $(CH_3)_3NCH_2CH_2O$-CDP	-	-
R-(-)-2-amino-1-propanol	2 uM[b]	2 uM
R-(-)-1-amino-2-propanol	10 uM	10 uM
S-(+)-2-amino-1-propanol	-	-
S-(+)-1-amino-2-propanol	10 uM	10 uM

a = biphasic curve peak at 3 uM
b = biphasic curve peak at 10 uM

These data taken together support the specificity of the effect of ethanolamine and ethanolamine-like compounds as defined in the present invention, on cholinergic neurons.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A pharmaceutical composition for the treatment of a patient having a neurological disorder asociated with dementia of the Alzheimer's type wherein said pharmaceutical composition comprises a pharmaceutically acceptable excipient and an effective amount of an active sterioisomeric form of a compound having the structure

$$R_1 - NH - \underset{R_2}{CH} - \underset{R_3}{CH} - R_4$$

or the pharmaceutically acceptable salt thereof,

wherein R_1 is hydrogen or a lower alkyl;

the R_2 and R_3 groups may be the same when hydrogen or different and are selected from the group consisting of hydrogen, the lower alkyls, and -COOM groups wherein M is hydrogen or a pharmaceutically acceptable cation; and

R_4 is selected from the group consisting of -OH, $-PO_3H_2$, $-OPO_3H_2$, cytidine 5'-diphosphate, and their pharmaceutically acceptable salts.

2. The composition of claim 1 wherein R_1 is hydrogen.

3. The composition of claim 1 wherein R_1 is a lower alkyl.

4. The composition of claims 1, 2 or 3 wherein R_2 and R_3 are both hydrogen.

5. The composition of claims 1, 2, 3 or 4 wherein R_4 is -OH.

6. The composition of claims 1, 2, 3 or 4 wherein R_4 is $-OPO_3H_2$.

7. The composition of claims 1, 2, 3 or 4 wherein R_4 is $-PO_3H_2$.

8. The composition of claims 1, 2, 3 or 4 wherein R_4 is cytidine 5'-diphosphate.

9. The composition of claim 1 wherein R_2 is -COOM and M is hydrogen or a pharmaceutically acceptable cation, R_3 is hydrogen and R_4 is -OH such that the compound is serine or a pharmaceutically acceptable salt thereof.

10. The composition of claim 5 wherein R_1 and R_2 are both hydrogen and R_3 is methyl.

11. The composition of claim 5 wherein R_2 is methyl, and R_1 and R_3 are both hydrogen.

12. The composition of claim 1 wherein the compound is selected from the group consisting of ethanolamine, phosphoethanolamine, CDP-ethanolamine, 2-(methylamino) ethanol, phosphonoethanolamine, R-(-)-2-amino-1-propanol, R-(-)-1-amino-2 propanol and S-(+)-1-amino-2-propanol.

13. A pharmacological precursor of the structural compound of the composition of claim 1 which can be administered to a human subject such that serine, ethanolamine, or a derivative thereof is released from said

precursor by normal biological processes after administration to the patient.

14. A method of treating a patient having a neurological disorder associated with dementia of the Alzheimer's type comprising administering to said patient an amount of the composition of claims 1 or 12 which composition is effective in amelioration of the dementia.

15. A method of treating a patient having a neurological disorder associated with dementia of the Alzheimer's type comprising administering to said patient an amount of the composition of claims 1 or 12 which is effective in increasing synthesis of acetyltransferase.

FIG. 1

C-CF (~1 - 1.3 kD)

SCHEMATIC OF PURIFICATION

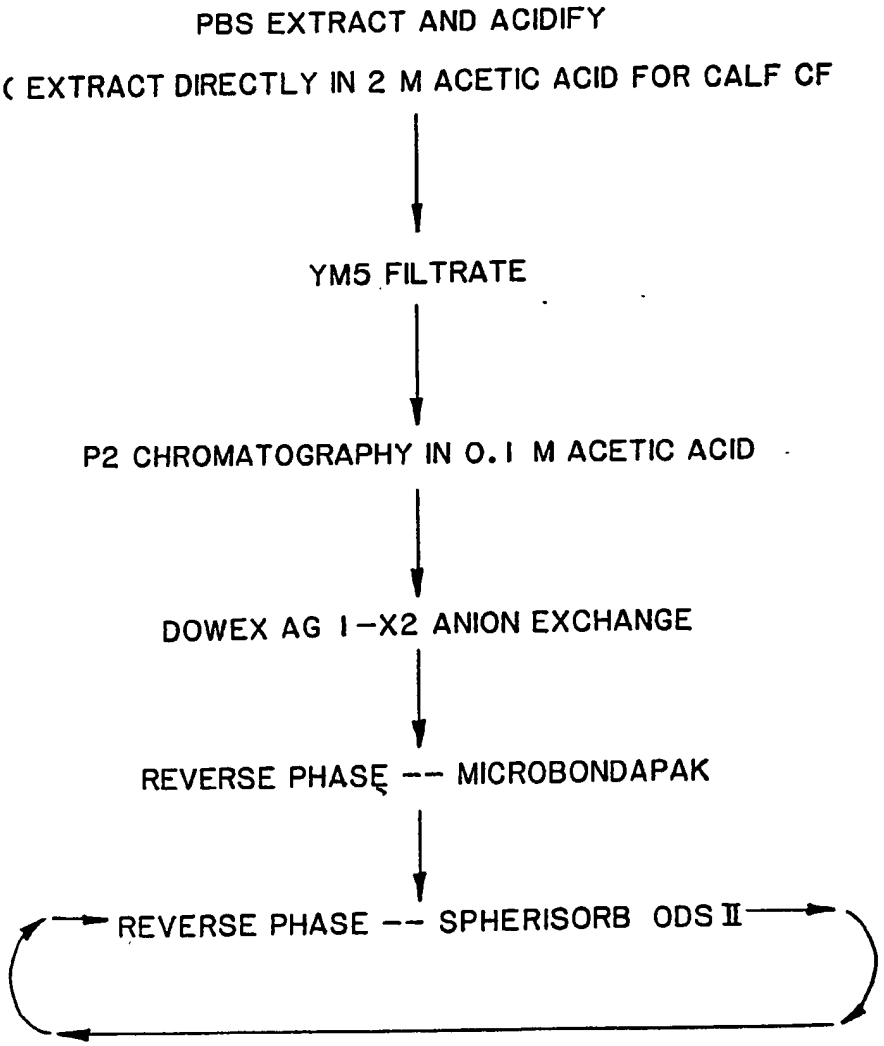
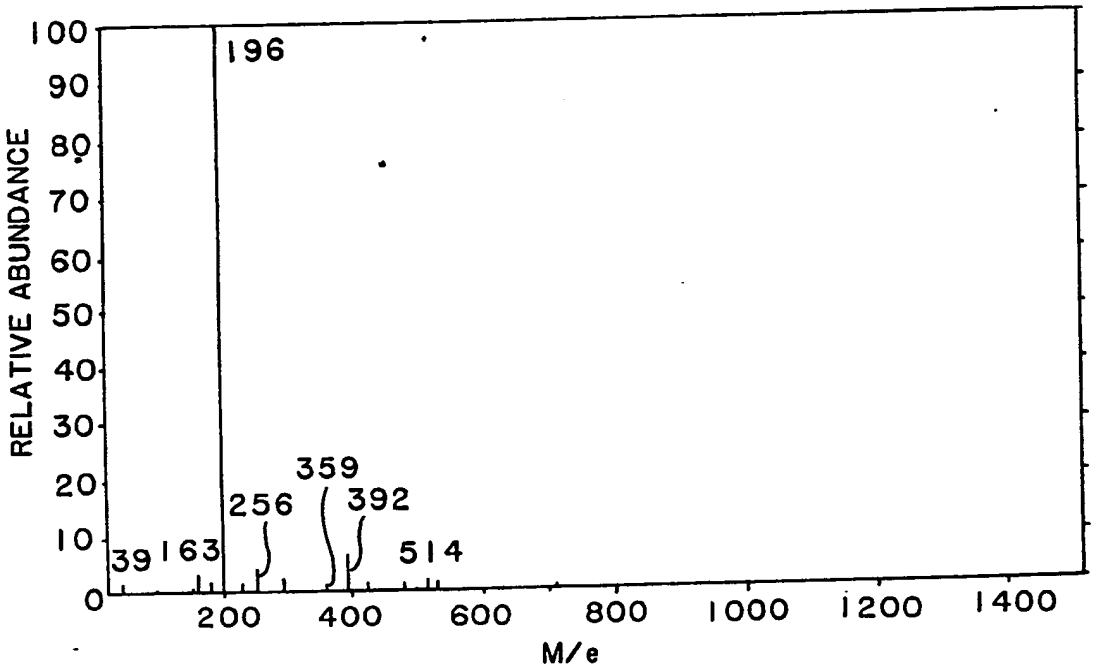
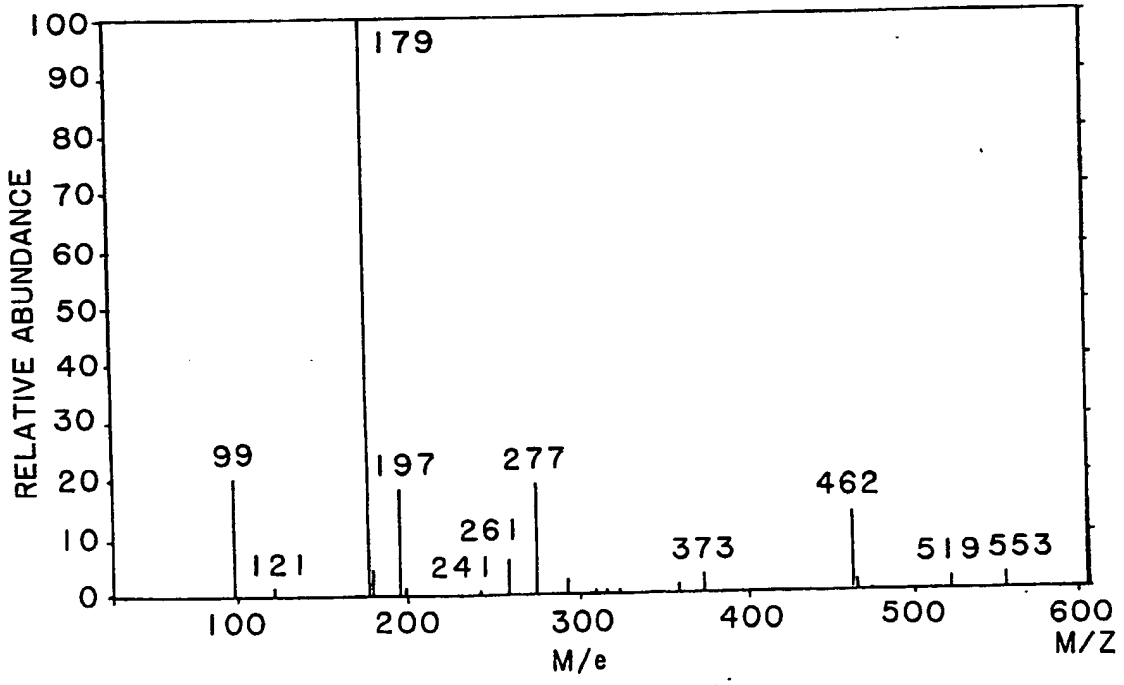


FIG. 2A



MASS SPECTRUM OF HYDROLYZED DERIVATIZED RAT C-CF

FIG. 2B



MASS SPECTRUM OF UNHYDROLYZED DERIVATIZED RAT C-CF

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 88/01693

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: A 61 K 31/13; 31/19; 31/195; 31/66; 31/70

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	A 61 K 31/00

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X,Y	FR, A, 2437834 (LEJEUNE) 30 April 1980, see the whole document, especially claims 1,3	1-12
Y	Fed. Proc. vol. 46, no. 3, 1987 L.C. Mokrasch: "Decreased transport of acetylcholine precursors into fibroplasts of Alzheimer's victims" see page 963, abstract no. 3769	
Y	EP, A, 0147185 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 3 July 1985, see page 5, lines 21-29; page 2, line 21 - page 3, line 19	1-12
Y	US, A, 4386078 (L.A. HORROCKS) 31 May 1983, see column 1, lines 14-64; claim 1	1-12
Y	Am. J. Psychiatry, vol. 138, no. 7, July 1981 M.Fisman et al.: "Double-blind trial of 2-dimethylaminoethanol in Alzheimer's disease", pages 970-972, see abstract; page 970, first paragraph	1-12

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
5th August 1988	- 6 SEP 1988

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

III. DOCUMENTS C NSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Neurology, vol. 34, suppl. 1, 1984 J.W. Pettegrew et al.: P-31 NMR changes in Alzheimer's and Huntington's disease brain", see page 281, abstract no. PP 317 --	1-12
X	Journal of Neurology, Neurosurgery, and Psychiatry, vol. 48, C.C.T. Smith et al.: "Putative amino acid transmitters in lumbar cerebrospinal fluid of patients with histologically verified Alzheimer's dementia", pages 469-471, see summary; page 470, table --	1-12
P,Y	Brain Research, vol. 417, 1987 D.W. Ellison et al.: "Phosphoethanol-amine and ethanolamine are decreased in Alzheimer's disease and Huntington's disease", pages 389-392, see abstract	1-12

FURTHER INFORMATION CONTINUED FR M THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 14-15 because they relate to subject matter not required to be searched by this Authority, namely:

PCT-Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods

2. [X] Claim numbers 13, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Reason.: A chemical compound cannot be characterized by terms such as "pharmacological precursor of" or "released from said precursor by normal biological processes.."

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 8801693

SA 22576

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 29/08/88
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
FR-A- 2437834	30-04-80	None	
EP-A- 0147185	03-07-85	JP-A- 60252416	13-12-85
		US-A- 4569929	11-02-86
US-A- 4386078	31-05-83	None	